

MEDROXYPROGESTERONE ACETATE HAS MINIMAL IMPACT ON HIV-1 REPLICATION IN ECTOCERVICAL TISSUE EX VIVO

by

Hawa Mariko

BS, Towson University, 2013

Submitted to the Graduate Faculty of
the Graduate School of Public Health in partial fulfillment
of the requirements for the degree of
Master of Public Health

University of Pittsburgh

2017

UNIVERSITY OF PITTSBURGH
GRADUATE SCHOOL OF PUBLIC HEALTH

This thesis was presented

by

Hawa Mariko

It was defended on

April 14, 2017

and approved by

Jeremy J. Martinson, DPhil, Assistant Professor, Department of Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh

Zandrea Ambrose, PhD, Associate Professor, Department of Medicine, Division of Infectious Diseases, School of Medicine, University of Pittsburgh

Robbie B. Mailliard, PhD, Assistant Professor, Department of Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh

Thesis Director: Charlene S. Dezzutti, PhD, Associate Professor, Department of Obstetrics, Gynecology & Reproductive Sciences, Magee-Women's Research Institute; Associate Professor, Department of Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh

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Hawa Mariko, MPH

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ABSTRACT

A link between use of the contraceptive depomedroxyprogesterone acetate and risk of HIV-1 acquisition has been demonstrated in epidemiologic studies. Peak medroxyprogesterone-17-acetate (MPA) levels occur 4 days post-injection with serum progestin levels of 2 nM. However, other studies have evaluated its in vitro effects at supraphysiologic concentrations resulting in immune modulation of epithelial cell lines and peripheral blood mononuclear cells. To formally evaluate MPA effects on HIV-1 infection in a more physiologic model, we utilized our polarized ectocervical tissue explant model. Our hypothesis is physiologic concentrations of MPA will not affect HIV-1 replication in ectocervical tissue ex vivo.

Human ectocervical tissue was processed into polarized explants. Explants were cultured alone (control) or with MPA in the basolateral compartment at physiological (0.05nM, 0.5nM, 1nM, 5nM) (n=17) or higher concentrations (500nM, 5μM, 500μM) (n=10) for 48 h, infected with either 5×10^2 or 5×10^3 TCID₅₀ HIV-1_{BaL} apically for 24 h, and then washed. Basolateral supernatants were collected and replenished with fresh medium containing MPA every 3-4 days up to 21 days. Supernatant collections at the 48 h time point (pre-infection) were tested for cytokine expression while all following supernatant collections post-infection were analyzed for viral p24_{gag} concentrations via ELISA. PBMCs were cultured for 72 hours in media containing

MPA at 5 μ M, 50 μ M, and 500 μ M (n=5), including a negative and activated control, then analyzed for antibodies via flow cytometry.

Viral replication over 21 days in the presence of supraphysiologic concentrations of MPA indicated suppression, while that in the presence of physiologic concentrations was not significantly different from the control. Evaluation of cytokine markers indicated a hormone dose-dependent relationship between decreasing MPA concentrations (500 μ M-5nM) and increasing expression of pro-inflammatory cytokines approaching levels of the control, which were significant for IL-12 (p40), IL6, and IL-8.

MPA does not significantly affect HIV-1 replication at physiologic concentrations but has an overall suppressive effect on replication at supraphysiologic concentrations in ectocervical tissue in cervical tissue ex vivo.

The public health significance of DMPA continuation requires balancing unintended pregnancy while diminishing HIV-1 acquisition risk, especially when the risks of infection do not outweigh the contraceptive benefits.

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1.0 INTRODUCTION

The use of progestin-based hormones as a therapeutic agent provides a range of benefits for women, including serving as an effective birth control option [1]. Despite this advantage, progestin-based hormones, in particular injectable depo-medroxyprogesterone acetate (DMPA), have a suspected epidemiologic risk between use and HIV-1 acquisition [2-4]. In several systematic reviews, DMPA users had higher incident HIV-1 seroconversion rates than other contraceptives users [2-4]. DMPA has also been linked with immunological suppression by diminishing systemic innate and adaptive immunity against HIV-1 replication or acting as a steroid inducing a local pro-inflammatory milieu [5-6]. Despite these concerns, use of DMPA is still recommended in resource poor countries where contraceptive choice is limited [7]. In Africa, and in particular southern Africa, injectable hormonal contraceptives have a high rate of use; in 2015, approximately 28.8% of modern contraceptive methods use was injectable [8]. Compounding this high usage rate, the region carries a high HIV-1 incidence rate of 0.56 in adults [9]. The coupling of a high percentage of injectable contraceptive users with high HIV-1 prevalence warrants further study on factors driving this epidemiologic relationship.

Of the two injectable progestin-based contraceptives, DMPA poses the greatest risk for HIV-1 acquisition while minimal risk was associated with norethisterone enanthate (NET-EN) use [10]. Female sex hormones influence all areas of the female genital tract, but the ectocervix or lower reproductive tract seems to be a preferred site for HIV-1 infection due to higher

abundance of immune cells, specifically CD4+ T cells, compared to vaginal epithelium [11-12]. While serum progestin levels peak at approximately 2nM early after dosage (approximately 4 days) [13], there is a long drug tail that could influence HIV-1 acquisition risk when serum levels reach steady state. Changes in viral chemokine co-receptor expression on T cells may also contribute to increased susceptibility. The expression of CCR5, the major HIV-1 coreceptor on T-cells, is positively associated with progestin-only contraceptive exposure [14-15]. In addition, CD4/CCR5 double-positive cervical T cells experience increased viral infection compared to T cell subsets from serum [16]. The higher rates of viral entry with cervical CCR5 combined with increased abundance in the presence of a progestin-based contraceptive possibly provides more target cells than otherwise would be found if no additional progestin were present, thereby facilitating infection. This indicates that DMPA influences the local mucosal immune profile in favor of HIV-1 infection. Furthermore, the steroidal activity of synthetic progesterone and its higher affinity to the glucocorticoid receptor (GR) than endogenous progesterone plays a role in its induction of pro-inflammatory mediators that may facilitate viral infection [17-19]. Ferreira et al. observed that HIV-1 exposed genital epithelial cells showed enhanced viral uptake and transcytosis in a dose-dependent manner in the presence of DMPA, but this work focused on high concentrations of hormone, ranging from 1nM to 100nM, which is above the peak physiological level [20]. In yet another in vitro model, a brief high-dose exposure (39-390µM) correlated with increased susceptibility to HIV-1 infection [21]. While this information suggests DMPA may increase HIV-1 susceptibility in individual epithelial or immune cell models, confirmation in a reproducible tissue model that contains the resident immune cells along with the intact epithelium and lamina propria instead of individual cells or cell lines is needed so mechanisms of MPA related to viral enhancement can be defined. The interplay between the

different cell types that are present in a tissue model would be allow for intercellular communication. Once established, mechanistic pathways can be pursued to understand how MPA may affect HIV-1 infection.

1.1 EPIDEMIOLOGIC ASSOCIATION

DMPA and NET-EN are both injectable, progestin-based hormonal contraceptives with differing associated observational risks for HIV-1 acquisition among users. While NET-EN portrays no significant effect, a meta-analysis of various contraceptive methods on HIV-1 acquisition shows that DMPA is more often significantly associated with increased risk but remains contradicted by data showing no effect [2]. Heffron et al. demonstrated a seroconversion incidence from HIV-1 negative women of 6.85 per 100 person-years for those using any hormonal injectable, compared to 3.78 for those not any hormonal contraception, and 5.94 for those on oral contraceptive [4]. This was supported by an adjusted hazard ratio of 2.05 for injectable hormonal contraceptives (95% CI 1.04-4.04). Furthermore, in a study evaluating the differential contributions of DMPA versus NET-EN in a group of women that used only one method, DMPA was associated with an adjusted hazard ratio of 1.29 for HIV-1 seroconversions compared to the NET-EN reference [10]. In a secondary analysis of women enrolled in the Methods for Improving Reproductive Health in Africa (MIRA) trial, both DMPA and NET-EN were not significantly associated with HIV-1 acquisition (HR 1.18, 95% CI 0.84-1.62; HR 1.40, 95% CI 0.72-2.35) even after adjustment for baseline characteristics, but overall, injectables showed significant association (HR 1.32, 95% CI 1.00-1.74) [25]. However, Kleinschmidt et al. showed no significant difference in HIV-1 acquisition for DMPA users nor NET-EN users compared to those not on

any contraceptive [26]. These discrepant results led the World Health Organization (WHO) to issue a recommendation in 2012 for HIV-1 negative women to implement male and female condom use or other preventive measures as a supplement to injectable progestin contraceptives [22]. An updated guideline recommendation in 2017 reaffirms a positive association between DMPA use and HIV-1 risk, but cites difficulties in teasing out, “causal relationships versus methodological limitations” [23].

1.2 MECHANISMS FOR HIV-1 ACQUISITION

1.2.1 Glucocorticoid activity

DMPA interacts with a wide range of steroid receptors aside from the progesterone receptor (PR) [27]. Compared to NET-EN and endogenous progesterone, DMPA exhibits higher affinities for the glucocorticoid receptor (GR), leads to greater GR phosphorylation, and has greater protection from ligand-receptor degradation [17]. DMPA also displays a higher affinity to the GR than the PR (while NET-EN demonstrates the reverse) and competitively binds with the corticosteroid, dexamethasone [17, 28]. GR engagement may result in transcription of related factors. In an evaluation of MPA on transcription of pro-inflammatory cytokine IL-12 and anti-inflammatory cytokine IL-10 in ectocervical cells, increasing concentrations of hormone resulted in increased expression of IL-12 and reduced expression of IL-10 via elevated recruitment of the GR to their respective promoter regions, compared to cortisol and endogenous progesterone [18, 29]. While this experiment was done using 1 μ M of hormone, physiologic concentrations of DMPA affect expression of pro-inflammatory genes in a dose-dependent manner; expression levels of IL-6 and

IL-8 in PBMCs gradually decreased as MPA concentrations increased from 1nM to 100nM [29]. PBMCs treated with concentrations of MPA at 10,000nM increased IFN- γ expression in the presence of a GR antagonist mifepristone compared to MPA lacking the antagonist, confirming that the inhibitory cytokine expression through high-dose MPA was mediated by engagement of the GR [43].

1.2.2 Local immunological environment

Synthetic progesterone exposure confers different chemokine/cytokine expression than endogenous progesterone exposure. DMPA use has been associated with significant upregulation of local female genital tract innate factors, specifically HNP1–3, LL-37, and lactoferrin; cationic polypeptides that have implications in recruitment of HIV-1 target cells [6, 30]. Although known to have some antiviral capabilities, HNP1 has been shown to facilitate viral migration across the epithelial barrier by disrupting tight junctions and increasing permeability [31-32]. LL-37 exhibits similar antiviral functions but also increases the expression of CD4 and CCR5 co-receptors in Langerhans Cells [33-34]. Furthermore, the chemokine/cytokine profile of cervicovaginal lavages from DMPA users resembles an inflammatory state, with an increase in the pro-inflammatory cytokines TNF- α , IL-1 α , IL-1 β , and IL-6, in addition to adaptive cytokines IL-2 and IFN- γ [30].

DMPA may facilitate HIV-1 infection by enhancing recruitment of activated target cells to the vaginal mucosa. There's still discrepancies in the literature as some have seen no change in CCR5 expression, especially one study where the percent CCR5 positive T cells in PBMCs from DMPA users didn't differ from other contraceptive groups [14], but another noted increased

expression of CCR5 after PBMCs were cultured in progesterone [15]. Even exposure to one dose of DMPA significantly increased T cell activation markers without significantly altering epithelial thickness [35]. Compared to the follicular phase, women who were given one 150mg dose of DMPA experienced increased levels of CD45, CD3, CD8, CD68, CCR5, and HLA-DR positive vaginal T cells measured at 12 weeks post-injection [35]. Interestingly, neither epithelial thickness nor E-cadherin levels (cell adhesion molecule) were significantly altered after DMPA treatment compared to the follicular and luteal phases, but the number of Ki-67 positive epithelial cells indicating proliferation was significantly increased after DMPA administration compared to both phases. This was attributed to the lack of subsequent dosing, leading to epithelial regeneration at 12 weeks post-dose. While no changes in epithelial integrity were noted in this experiment, DMPA exposure was associated with increased recruitment of target cells to the vaginal mucosa.

Synthetic progesterone in the context of herpes simplex virus-2 (HSV-2) infection plays a role in driving the MPA-HIV relationship. South African countries with high HIV-1 prevalence are also associated with having a high prevalence of HSV-2; the population attributable fraction (PAF) of new HIV-1 infections attributable to HSV-2 is estimated to be 35-50% [46]. Given this association, HSV-2 could be a trigger enhancing HIV-1 acquisition. A disrupted mucosal permeability resulting from MPA exposure leaves the vaginal mucosa permissive to HSV-2 infection [36], which also has been demonstrated to induce inflammation at the vaginal mucosa [37]. In a study evaluating T-cell activation and T-cell subset numbers in cervical cytobrush samples of women, HSV-2 positivity was associated with increased expression of CD3/CD4/CCR5 positive and CD69 positive cells compared to HSV-2 negative samples [37]. In HSV-2/HIV-1 co-infected ectocervical tissue models, the HSV-2/HIV-1 combination portrayed

significantly greater relative CD4/CCR5 expression than HIV-1 alone, and produced significantly greater HIV-1 replication [38]. Pre-existing HSV-2 infections create an inflammatory environment susceptible to HIV-1 infiltration and enhanced replication [39], and are heightened with MPA exposure. This is supported epidemiologically as HSV-2 seropositive women are twice as likely to seroconvert to HIV-1 when using DMPA, compared to NET-EN [10]. However, there remains a gap in our knowledge on the direct effects of MPA on HIV-1 transmission. Our hypothesis is that physiological levels of MPA will not affect HIV-1 infection in mucosal tissue *ex vivo*.

1.3 EXPERIMENTAL RATIONALE

To characterize the impact exogenous progestins may have on genital HIV-1 transmission, polarized ectocervical tissues were treated with sustained concentrations of MPA and exposed to HIV-1 and changes in HIV-1 replication and soluble immune mediators were measured. Because of logistical concerns of isolating immune cells from tissue, peripheral blood mononuclear cells (PBMCs) were treated with physiologic and supraphysiologic concentrations of MPA to understand its effect on CD4⁺ T-cell surface markers that are important for cellular activation and HIV-1 infection. Given the implications of the undescribed long-term effects of DMPA combined with previous experiments at various hormone concentrations, we hypothesized that physiologic concentrations of DMPA will not affect HIV-1 replication.

The public health consequence of DMPA continuation requires balancing unintended pregnancy while diminishing HIV-1 acquisition risk. Complete discontinuation of DMPA is not

recommended as currently the risks of infection do not outweigh the contraceptive benefits, especially when compared to relatively less effective methods, condoms alone or oral contraceptives [22-24]. Furthermore, it is estimated that a shift from DMPA to oral contraceptives would result in 165 additional unwanted births per 100 HIV-1 infections averted among serodiscordant couples, but in the same high-risk population would peak to 1276 additional unwanted births per 100 HIV-1 infections averted if switched to no contraceptive method [24]. More research investigating biological mechanisms and linking with behavioral/environmental factors is necessary to identify drivers of the DMPA-HIV relationship.

2.0 MATERIALS AND METHODS

2.1 REAGENT PREPARATION

Unless otherwise stated, all culture media was obtained from Cellgro (Manassas, VA), all media supplements were obtained from Gemini BioProducts (West Sacramento, CA), and all antibodies for flow cytometry were obtained from BD BioSciences (San Diego, CA).

2.2 EX VIVO ECTOCERVICAL TISSUE CULTURE

Normal cervical tissue was obtained from the University of Pittsburgh Health Sciences Tissue Bank (Pittsburgh, PA) via surgical resection from women undergoing routine hysterectomies (IRB PRO09110431), or procured from a donor tissue to the National Disease Research Interchange (NDRI, Philadelphia, PA). The median age of donors was 42 years old (range 27-50).

Cervical tissue specimens were processed into explants of 5mm in diameter of epithelium and approximately 4mm in thickness encompassing the lamina propria. All experiments were performed in duplicate. Explants were polarized for culture on a 12mm transwell insert with the epithelial layer oriented apically, the basolateral layer oriented basally, and the stromal edges sealed by MatrigelTM Basement Membrane Matrix (BD BioSciences, San Jose, CA).

2.3 HORMONE PREPARATION

Medroxyprogesterone 17-acetate (MPA; Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50,000 μ M and stored in aliquots at -20°C until ready for use. Explants were cultured for 48 hours at 37°C 5% CO₂ in phenol-red free Dulbecco's Modification of Eagle's Medium cervical media supplemented with 10% charcoal-treated human A/B serum (to control for exogenous hormone exposure), 1% Penicillin/Streptomycin/L-glutamine, and 1% nonessential amino acids in the basolateral compartment of the transwell exposing only the explant's basolateral layer to hormone. Controls were cultured in media without MPA, while other explants were cultured with the indicated MPA concentrations. The explant culture supernatants were collected from the basolateral compartment at the 48-hour time-point then and stored at -80°C.

2.4 HIV-1 INFECTION

After the 48 hours, each explant was infected apically with either 5 \times 10² or 5 \times 10³ TCID₅₀ (tissue culture infectious dose for 50% infectivity) HIV-1_{BaL} and incubated for 24 hours at 37°C 5% CO₂, then washed with an isotonic saline solution to remove excess virus. Basolateral supernatants were collected again after 24 hours, and replenished with fresh medium containing equivalent amounts of MPA every 3-4 days up to 21 days of culture. Supernatant collections were kept frozen at -80°C until p24 could be quantified using the Alliance HIV-1 enzyme-linked immunosorbent assay (ELISA) kit (Perkin Elmer, Waltham, MA).

2.5 CYTOKINE EVALUATION

Basolateral supernatant collections after explant incubation for 48 hours in the designated control media, or MPA-containing media were evaluated for cytokine analyte expression using the Milliplex Human Cytokine/Chemokine 9-plex Magnetic Bead Kit (Millipore Corp., St. Charles, MO). All samples were run in duplicate for the following analytes: GM-CSF, IFN- γ , IL-12 (p40), IL-1 β , IL-6, IL-8, IP-10, MIP-1 β , TNF- α . Expression was measured using the Luminex MAGPIX system with xPONENT software V4.2.1324.0 (Luminex Corporation, Austin, TX).

2.6 PBMC PREPARATION

Whole blood was obtained from Central Blood Bank (Pittsburgh, PA) then processed for isolation of peripheral blood mononuclear cells (PBMCs). PBMCs were incubated at 2×10^6 cells/mL for 72 hours at 37°C 5% CO₂ in phenol-red free RPMI-1640 PBMC medium supplemented with 10% heat-inactivated charcoal-treated fetal bovine serum (FBS) and 1% Penicillin/Streptomycin/L-glutamine, plus one of the following treatments: a negative control, a positive control containing 1,000 U IL-2 and 5 μ g/mL PHA-P, or MPA at the indicated concentrations. After 72 hours, PBMCs were washed for flow cytometry at an optimal concentration of 6-12 $\times 10^6$ cells/mL.

2.7 FLOW CYTOMETRY

Cells were prepared for flow cytometry analysis of surface protein expression using the following antibodies: CD3 – Pacific Blue, CD4 – Alexa Fluor 700, CCR5 – PE, CXCR4 – PerCP Cy 5.5, CD69 – PE-Cy7, HLA-DR – FITC, $\alpha 4\beta 7$ – APC (BD BioSciences, San Jose, CA). Viability staining was done using LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit, for 405 nm excitation (Invitrogen/ThermoFisher, Eugene, OR). Cell surface expression of antibodies was performed using a BD LSRII flow cytometer with 10,000 events per sample (BD BioSciences, San Jose, CA). Cell populations were gated from live, single cell population using fluorescence minus one (FMO) strategy (Figure 1). Data was extrapolated in FlowJo software (TreeStar Ashland, OR).

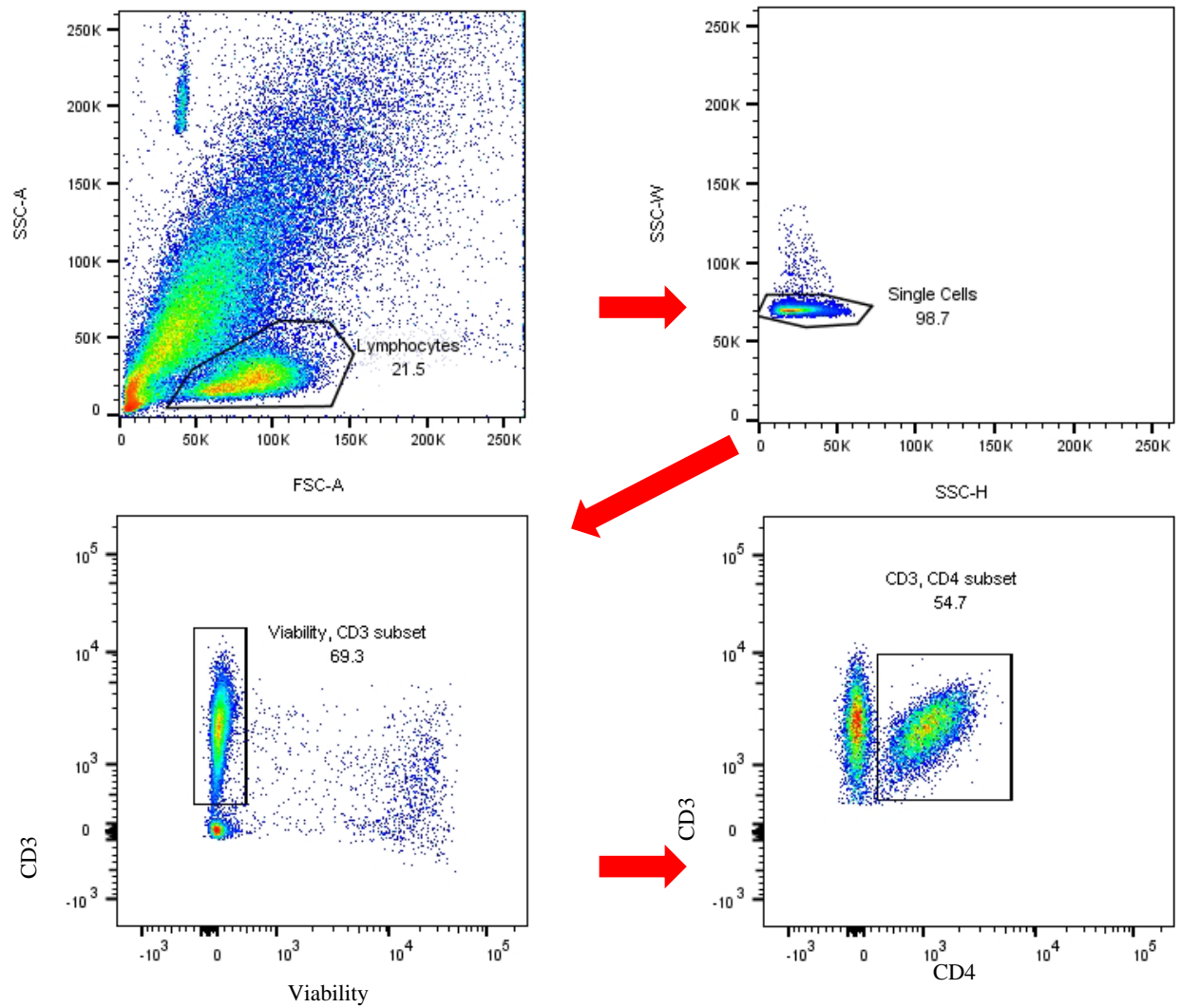


Figure 1. Flow cytometry gating strategy

Live, single cell lymphocyte populations were gated using fluorescence minus one (FMO) strategy to isolate viable CD3+CD4+ double positive cells.

2.8 STATISTICAL ANALYSES

Cumulative p24 was calculated using the Virus Endpoint Calculator (AlphaStatConsult LLC, Damascus, Maryland) and compared between groups using a nonparametric Kruskal-Wallis test

and post-hoc Dunn's test. Significance was defined as $\alpha=0.05$ in GraphPad Prism 5 (GraphPad Software, San Diego California USA, www.graphpad.com).

Flow cytometry analysis was performed using Kruskal-Wallis test and post-hoc Dunn's test to compare median differences between percent positive cell populations. All flow cytometry statistical analysis was performed in GraphPad Prism 5.

Median differences in cytokine marker expression between treatment groups was analyzed by Generalized Estimating Equations (GEE) analysis to the untreated control in STATA/SE 14 significance level $\alpha=0.05$.

A linear mixed effects model with random intercepts was applied to compare p24 output between treatment groups over the course of 21 days. The amount of p24 was defined as the continuous outcome, hormone concentration was defined as a fixed effect, day of collection was defined as a repeated measure, and each tissue was defined as a random factor. Separate models were applied to each viral titer. A Test of Fixed Effects was applied to evaluate the significance of fixed effects on the outcome in STATA/SE 14 significance level $\alpha=0.05$.

3.0 RESULTS

3.1 LOW-DOSE MPA EXPOSURE SUGGESTS VIRAL ENHANCEMENT, WHILE HIGH-DOSE MPA EXPOSURE SUGGESTS VIRAL SUPPRESSION

3.1.1 Viral replication

Polarized ectocervical tissue explants were treated with MPA concentrations (0.05nM-500μM) and subsequently infected with HIV-1 to determine if exogenous progestins impact viral replication. Viral replication was similar over the course of 21 days and was independent of viral infectious titer, where supraphysiologic concentrations of MPA suppressed viral replication and physiologic concentrations of MPA demonstrated a slight viral enhancement as compared to the untreated control explants. The amount of p24 measured for tissues treated with the lowest MPA concentrations (0.05nM-5nM) and infected with a 5×10^3 TCID₅₀ of HIV-1 produced more or equivocal amounts of virus compared to the MPA-free control throughout the duration of culture (Figure 2A). Similarly, p24 output for tissues treated with the highest concentrations of MPA (5μM-500μM) remained below the control for 21 days of culture (Figure 2B). When explants were infected with a 5×10^2 TCID₅₀ viral titer, the difference in viral replication was more defined. Viral output for tissues treated with 5nM-500nM of MPA remained above the MPA-free control, while tissues treated with concentrations well above physiologic levels (5μM-500μM)

displayed less viral replication compared to the control (Figure 2C). Overall for both viral titers, there was a consistent pattern of viral suppression compared to the control for supraphysiologic concentrations of hormone at 500 μ M and 5 μ M. In addition, lower concentrations of MPA at 5nM and below physiologic concentrations showed slight viral enhancement compared to the control.

Stock hormone was dissolved in DMSO, added to cervical media to make the 500 μ M MPA solution, and then serially diluted in cervical media to achieve lower hormone concentrations. We evaluated the effects of DMSO on viral growth by including a DMSO control, where it was added to cervical media (without MPA) at concentrations comparable to the amount of DMSO used for the 500 μ M MPA. We noticed minimal HIV-1 replication in the DMSO control compared to the negative control, and that resembled viral growth at the 500 μ M MPA concentration (Appendix Figure 1 and 2). Although only one tissue was used for this evaluation thus far (n=1), this suggests DMSO may influence replication as lower concentrations of hormone contain successively less DMSO than the preceding concentration. Improvements to this model would include verifying that all hormone concentrations also contain the same amount of DMSO.

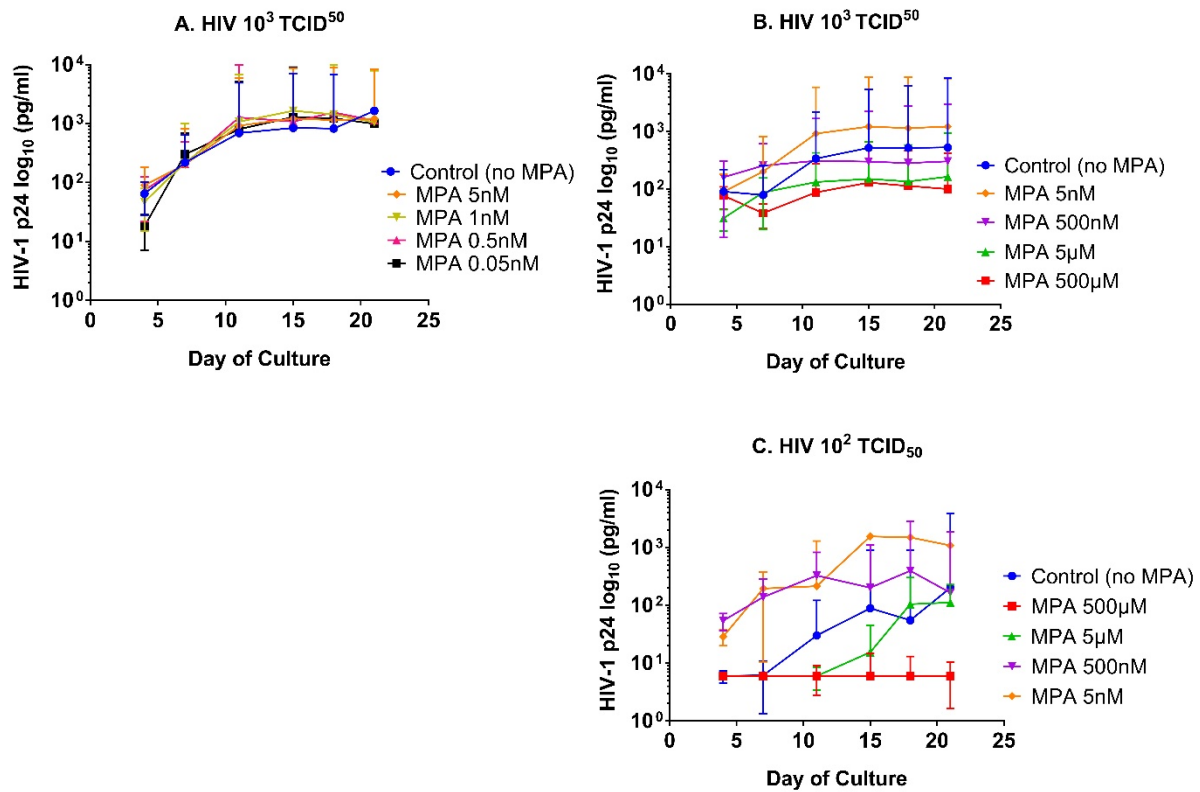


Figure 2. Viral growth and 95% CI

Viral growth over time is represented by median concentrations of p24 in explant supernatant collected after incubation for 48 hours with either 500μM, 5μM, 500nM, 5nM, 1nM, 0.5nM, or 0.05nM MPA, then infected with (A) 5x10³ TCID₅₀ HIV-1_{BaL} (n=5), (B) 5x10³ TCID₅₀ HIV-1_{BaL} (n=12), or, (c) 5x10² TCID₅₀ HIV-1_{BaL} (n=10). Error bars represent the 95% CI.

3.1.2 Cumulative viral growth

A different analysis comparing cumulative p24 produced during culture against other treatment groups recapitulates the results using the raw HIV-1 replication curves (Figure 3). There was a negative trend of decreasing viral replication with increasing MPA concentration for both viral infection titers. Tissues treated with 5nM and infected with 5x10² TCID₅₀ HIV-1_{BaL} showed greater absolute differences in median cumulative p24 compared to those infected with 5x10³

TCID₅₀ HIV-1_{BaL} (Figure 3A and 3B). Interestingly, explants treated with 500nM MPA suggested viral enhancement when infected with a lower viral titer (Figure 3A), but suppression when infected with a higher viral titer (Figure 3B). Higher concentrations of hormone at 5μM and 500μM portrayed less cumulative p24, again suggesting viral suppression while lower concentrations remained above or near the median control for the lowest concentrations of MPA (Figure 3A and 3B). Overall significance was only detected for the 5×10^2 TCID₅₀ infections (Figure 3A, P=0.0125), but could not be attributed to any single pairwise comparison. Physiologic concentrations of hormone did not have an effect on viral replication (Figure 3C, P=0.0670). Overall, the differences in cumulative p24 were not significant for the 5×10^3 TCID₅₀ infections (Figure 3B, P=0.7932; Figure 3C, P=0.0670).

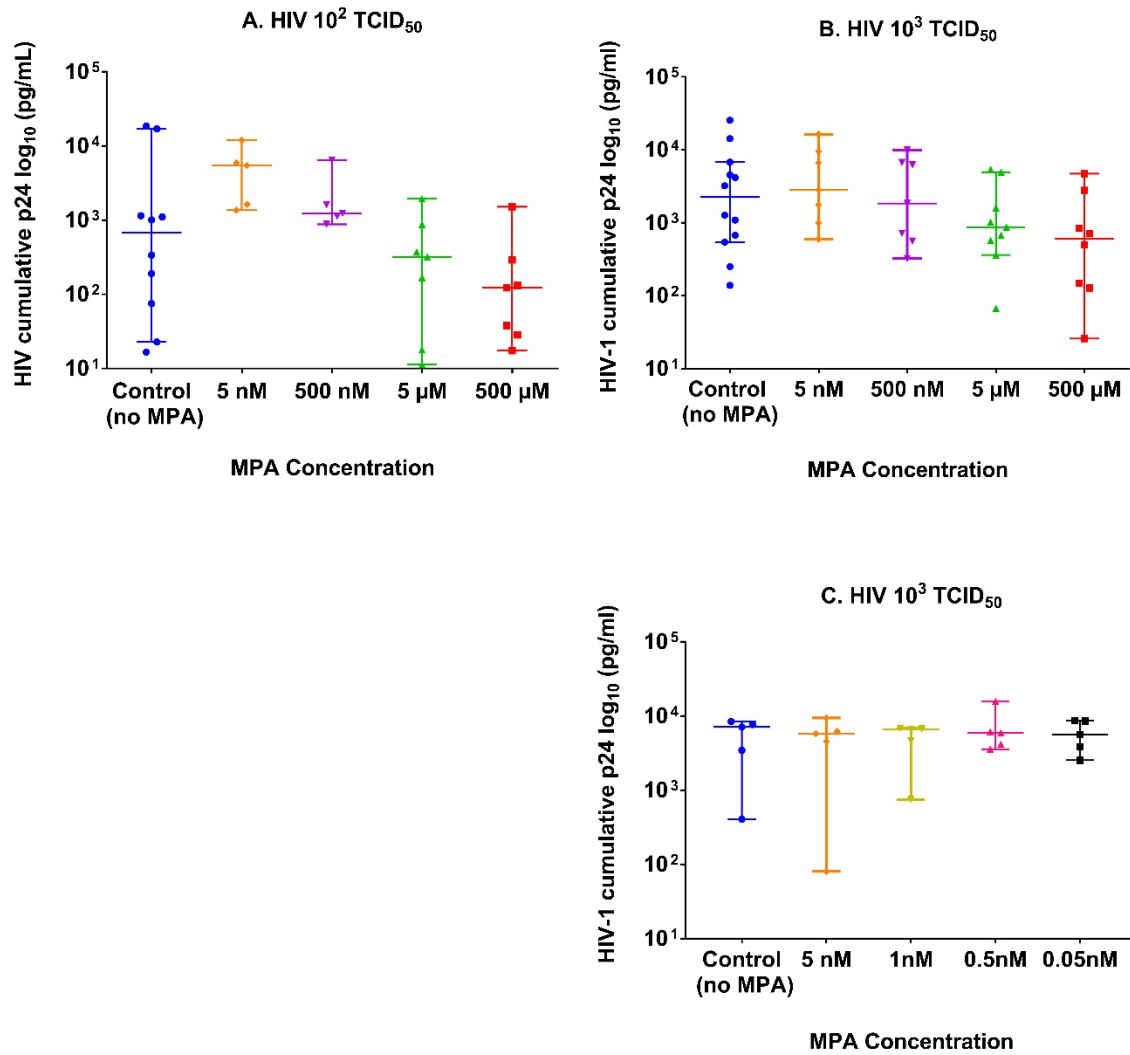


Figure 3. Cumulative p24

Cumulative p24 is represented for 6 collection days after incubation for 48 hours with either 500 μ M, 5 μ M, 500nM, 5nM, 1nM, 0.5nM, or 0.05nM MPA, then infected with (A) 5×10^2 TCID₅₀ HIV-1_{BaL} (n=10), (B) 5×10^3 TCID₅₀ HIV-1_{BaL} (n=12), or (C) 5×10^3 TCID₅₀ HIV-1_{BaL} (n=5). Cumulative median p24 was compared to the control group using a Kruskal-Wallis ANOVA, and pairwise comparisons made to the Control group using Dunn's test. Error bars represent the 95% CI.

3.1.3 Linear mixed effects model

An evaluation of HIV-1 replication over time compared to a given MPA concentration portrayed a trend of suppression in viral growth for higher concentrations of MPA (Table 1). In a linear mixed effects model supraphysiologic concentrations (500 μ M) portrayed significant viral suppression over time shown by a negative difference in the slope compared to the control ($P=0.025$, $P<0.001$). The lowest concentration of MPA (5nM) portrayed significant viral enhancement over time only for the lower titer (5×10^2 TCID₅₀ HIV-1_{BaL}, $P=0.005$). In support of the 5×10^2 TCID₅₀ viral replication (Figure 1A and 1B), tissues treated with a physiologic concentration of MPA (5nM) had greater predicted p24 values compared to the untreated tissues. For a given low MPA concentration (0.05nM-5nM), there was a trend of viral enhancement indicated by positive, increasing slopes as hormone concentration decreases, although none were significant. Using the 5×10^2 TCID₅₀ HIV-1 may show a better association between physiologic MPA concentrations and viral enhancement.

Table 1. Viral growth in cervical tissue treated with medroxyprogesterone acetate (MPA)

	Treatment group	Slope difference (Δ)	P-value
5×10^2 TCID₅₀HIV-1_{BaL} (n=10)			
	MPA 500 μ M	-0.618	P=0.025*
	MPA 5 μ M	-0.375	P=0.173
	MPA 500nM	+0.205	P=0.519
	MPA 5nM	+0.899	P=0.005*
5×10^3 TCID₅₀HIV-1_{BaL} (n=12)			
	MPA 500 μ M	-1.264	P<0.001*
	MPA 5 μ M	-0.645	P=0.003*
	MPA 500nM	-0.212	P=0.355
	MPA 5nM	+0.285	P=0.214
5×10^3 TCID₅₀HIV-1_{BaL} (n=5)			
	MPA 5nM	-0.068	P=0.820
	MPA 1nM	+0.416	P=0.166
	MPA 0.5nM	+0.484	P=0.107
	MPA 0.05nM	+0.526	P=0.080

In a linear mixed effects model analyzing viral growth over time for a given MPA concentration, supraphysiologic concentrations (500 μ M, 5 μ M) again portrayed significant suppression over time shown by a negative significant difference in the estimate of the fixed effect compared to the control. In addition, the lower concentrations of hormone trended towards enhancement of viral replication over time (for a higher viral titer).

3.2 CYTOKINE EXPRESSION

The cytokine profile was analyzed using supernatants collected from pre-infected tissues cultured in their respective supraphysiologic MPA-containing media for 48 hours, a decrease in pro-inflammatory cytokines compared to the negative control (Table 3) was observed. For the analytes, there was a trend of decreasing cytokine expression as MPA concentration increased (Figure 4). Expression levels of IL-12, IL-6, and IL-8 indicate significant dose-dependent suppression (Table 3; P<0.005, P<0.05). Supraphysiologic MPA concentrations pointed to immunological suppression but as it approached more physiologic MPA concentrations

(0.05nM) the suppressive effects diminished. Interestingly, IP-10 expression levels only peaked at $1,041.32 \pm 1,074.92$ pg/mL for 1nM of MPA (Table 3). In addition, levels of GM-CSF did have an inverse trend but only pointed to significant upregulation at physiologic concentrations. GM-CSF expression at 1nM and 0.05nM were significantly greater than the control, while that at 0.5nM was not (Table 3).

Table 2. Median cytokine production from cervical tissue treated with medroxyprogesterone acetate (MPA)

	Cytokine Marker (pg/mL \pm standard deviation)								
	GM-CSF	IFN- γ	IL-12 (p40)	IL-1 β	IL-6	IL-8	IP-10	MIP-1 β	TNF α
Control (no PHA-P / IL-2) (n=10)	11.93 \pm17.35	13.60 \pm10.47	16.16 \pm11.04	2.47 \pm3.00	16,728.83 \pm13,607.76	48,321.72 \pm54,587.44	207.61 \pm5,242.71	41.02 \pm36.07	5.65 \pm5.72
MPA 500μM (n=6)	9.13 \pm 4.67	3.77 \pm 0.84	*** 6.55 \pm 3.22	** 1.08 \pm 0.52	*** 5,422.40 \pm 2,120.70	*** 12,567.65 \pm 6,845.53	36.19 \pm 57.57	16.12 \pm 16.06	3.89 \pm 1.23
MPA 5μM (n=7)	10.47 \pm 9.83	4.25 \pm 76.30	** 8.57 \pm 4.32	1.79 \pm 5.62	** 5,443.38 \pm 5,918.30	*** 18,307.99 \pm 14,912.19	34.12 \pm 8,771.21	21.00 \pm 164.22	3.89 \pm 11.52
MPA 500nM (n=7)	8.08 \pm 2.62	6.31 \pm 4.45	*** 8.43 \pm 4.12	1.64 \pm 0.61	** 5,095.41 \pm 6,189.42	*** 18,631.52 \pm 12,420.24	54.72 \pm 459.06	23.83 \pm 17.51	3.64 \pm 1.28
MPA 5nM (n=9)	10.76 \pm 7.37	6.93 \pm 5.23	15.84 \pm 11.69	2.65 \pm 1.08	*** 9,674.22 \pm 11,239.56	*** 26,905.63 \pm 42,809.81	71.37 \pm 1,887.41	31.00 \pm 20.40	4.79 \pm 3.97
MPA 1nM (n=3)	** 12.36 \pm 2.36	16.66 \pm 6.66	30.35 \pm 0.35	3.68 \pm .685	24,803.06 \pm 4,803.061	*** 69,380.63 \pm 9,380.631	1,041.32 \pm ,041.323	69.50 \pm 9.50.	16.42 \pm 6.42

Table 2 Continued

MPA 0.5nM (n=3)	18.87 ±31.46	14.45 ±11.79	30.34 ±4.01	4.36 ±1.72	34,350.70 ±21,897.02	*** 84,752.75 ±51,274.47	538.61 ±305.59	69.26 ±24.27	14.83 ±8.49
MPA 0.05nM (n=3)	** 44.72 ±32.20	17.16 ±7.60	31.07 ±2.53	43.78 ±0.70	25,720.46 ±12,863.82	*** 88,723.13 ±36,457.71	799.12 ±220.00	54.35 ±9.87	11.10 ±4.08
Summary of median cytokine values (pg/mL) and standard deviation from the 48-hour time point collections, before infect. P-values are representative of concentration group comparisons to the control in GEE analysis. ** P<0.05, ***P<0.005									

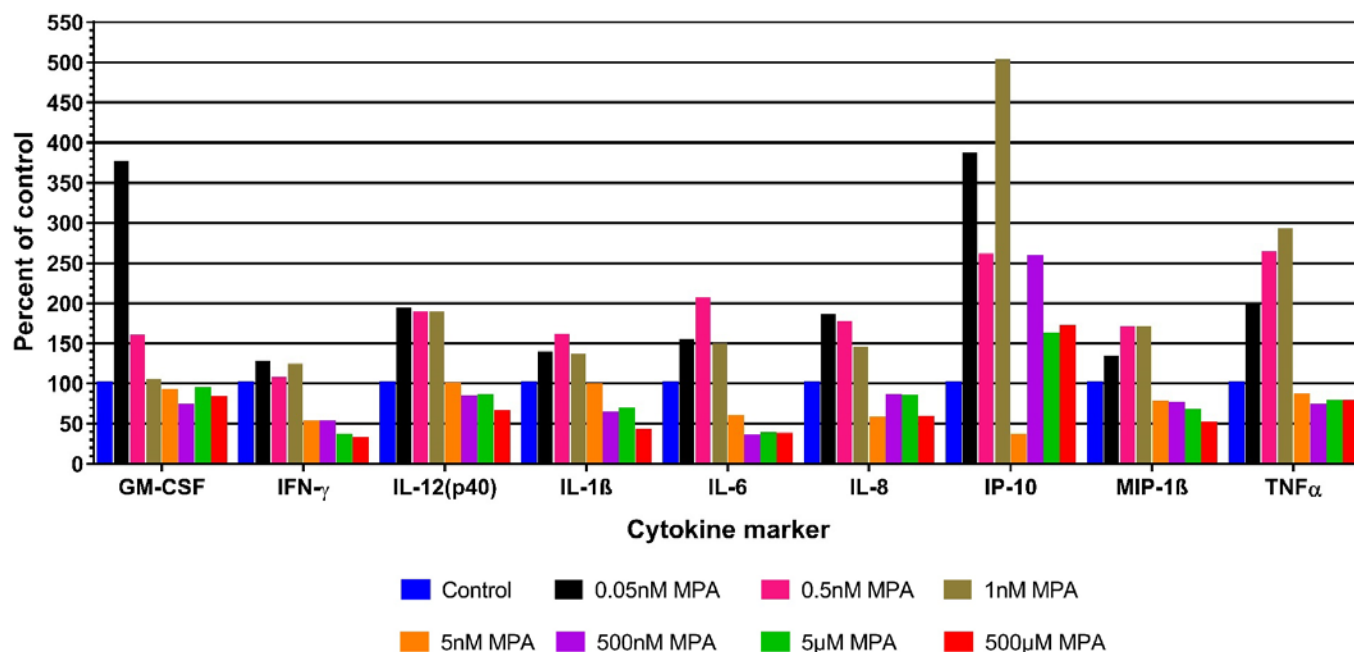


Figure 4. Cytokine production from ectocervical tissue

Cytokine production from cervical tissues treated with Medroxyprogesterone acetate (MPA). The data shown are the percent of control.

3.3 HIGH-DOSE MPA IS ASSOCIATED WITH IMMUNE SUPPRESSION

To define changes in cell activation and HIV-1 receptor expression on T cells, PBMCs were utilized initially rather than tissue to evaluate several \log_{10} concentrations of MPA. Once identified, tissue will be treated with those MPA concentrations to determine if tissue-associated immune cells respond in a consistent manner. An investigation of T cell surface markers after PBMC treatment with high-dose MPA shows immune suppression with the reduction in activation. Compared to the negative, non-activated control, MPA at 50μM and 500μM decreased the percentage of CD4⁺ T cells that were positive for HLA-DR (4.04%±2.49 versus

1.06%±1.65 and 1.41% ± 0.63, respectively) (Table 2). While the differences were small and not significant, there was a 3.4-fold increase in the percentage of CD3+/CD4+ T cells positive for CCR5 when treated with 5μM MPA as compared with the negative control (Table 2).

Table 3. Peripheral blood T cell marker expression after treatment with medroxyprogesterone acetate (MPA)

	Percent of live, CD3+ cells	Percent of CD4+ T cells				
Sample (n=5)	%CD4+	% CCR5+	% CD69+	% CXCR4+	% HLA-DR+	% α4β7+
Control (no PHA-P / IL-2)	31.80% ± 11.61	0.13% ± 0.14	5.33% ± 3.77	97.7% ± 4.36	4.04% ± 2.49	12.50% ± 3.83
Control (with PHA-P/IL-2)	12.80% ± 11.77	0.08% ± 0.10	61.30% ± 4.03	92.90% ± 17.65	30.60% ± 9.73	8.40% ± 3.74
MPA 5μM	35.90% ± 19.34	0.45% ± 0.64	7.93% ± 6.43	93.80% ± 9.02	3.03% ± 2.10	8.22% ± 1.97
MPA 50μM	44.60% ± 15.93	0.19% ± 0.13	5.73% ± 6.11	93.50% ± 4.84	1.06% ± 1.65	7.37% ± 1.63
MPA 500μM	44.60% ± 16.60	0.22% ± 0.12	8.78% ± 6.08	92.60% ± 2.05	1.41% ± 0.63	7.32% ± 1.94
<i>P-values</i>	0.5493	0.1126	0.4489	0.2049	0.1378	0.7380
Median percent positive of parent populations with standard deviation. A Kruskal-Wallis ANOVA compared medians of each surface marker group to the negative control. The activated control with PHA-P/IL-2 was not included in the statistical analysis.						

4.0 DISCUSSION

Low, physiologic concentrations of DMPA did not significantly affect HIV-1 replication over time in ectocervical tissue *ex vivo*, while high concentrations that are not representative of physiologic levels suppressed HIV-1 replication. Tissues treated with supraphysiologic concentrations of MPA had reduced production of pro-inflammatory cytokines as well. PBMC treatments with similar high concentrations of MPA decreased expression of cellular activation markers. These data suggest that supraphysiologic concentrations of MPA likely act through the GR causing immune suppression while physiological concentrations of MPA do not.

Despite the suggestive epidemiologic association between DMPA use and HIV-1 acquisition, an updated WHO guideline reiterates the use of DMPA in resource poor countries [23]. While many cite that discontinuation of DMPA without a comparable replacement in at-risk populations would impose more harm than benefit by increasing mother/child morbidity and mortality [23-24], identifying the factors that contribute to the relationship would decrease HIV-1 incidence from the appropriate source. Furthermore, methodology from secondary analyses has limitations on the association of DMPA on HIV-1 risk, for example self-reported usage, inconsistent hormone exposures among users (i.e. contraceptive switching), unspecified DMPA formulation types (intramuscular dosage versus subcutaneous dosage), controlling for, “time-varying confounders,” (i.e. coital frequency, condom use frequency), or controlling for personal exposure risk to HIV-1 [40]. These variables may confound the effect DMPA has on acquisition

risk. The Evidence for Contraceptive Options and HIV Outcomes (ECHO) trial is projected to complete data collection in 2019 and prospectively evaluates HIV-1 incidence between four randomized arms of contraceptive use, including DMPA, levonorgestrel implant (a long-acting reversible progestin-based implant), and copper IUD [41]. Results from this trial will supplement current literature by prospectively investigating risk (rather than secondary analyses) while controlling for age, contraceptive switching, HSV-2 status, and disease progression in HIV-1 seroconverts.

As serum progestin levels peak to 2nM approximately 4 days after dosage [13], the risk association noted in observational studies could point to the long drug tail as a window of infection opportunity. Supraphysiologic doses of hormone (500nM to 500µM) showed HIV-1 suppression over time and led to a reduction in pro-inflammatory chemokine/cytokine expression and CD4+ T cell activation markers. The use of DMSO, which can be toxic in tissues at high doses as used in the supraphysiologic MPA doses, for hormone dilutions also appeared to suppress HIV-1 replication but will require additional testing for a complete evaluation. When MPA was applied to the ectocervical tissue at physiologic concentrations (0.05nM to 5nM), trends toward increased HIV-1 replication was noted as seen with higher p24 levels when compared to the untreated control tissue. These data are consistent with relative HIV-1 infection differences noted in non-activated PBMCs treated with MPA ranging from 0.007nM-7nM [42]. While differences in HIV-1 infection of the PBMCs were significantly enhanced at physiologic concentrations of MPA (approximately 5.0 times greater at 0.7nM than the MPA-free control), initial infection was done using 100ng of p24 and using whole PBMCs instead of vaginal or cervical-sourced cells or tissue [42]. The results presented here suggest the minimal increase in viral replication at physiologic concentrations does not support MPA as the sole cause of

increased HIV-1 susceptibility. In conjunction with the viral infection data, no significant changes in HIV-1 coreceptors such as CCR5 or CXCR4 and cell activation markers were noted in PBMCs evaluated here. It would be of interest to investigate physiologic MPA concentrations, which resulted in modest increases in HIV-1 infection of ectocervical tissue, on the impact of mucosal T cell expression of CCR5 and their activation.

Pro-inflammatory cytokine expression exhibited a dose-dependent pattern where increasing hormone concentrations above physiological concentrations were associated with decreasing expression of pro-inflammatory cytokines compared to the control. These data were consistent with previous gradual relative suppression of IL-6 and IL-8 [38] ranging from 0.1nM MPA to 100nM MPA, whereas supraphysiologic concentration of 100nM and 1000nM were significantly decreased compared to the 1nM reference dose [39]. The opposite effect was seen for relative expression levels in vaginal epithelial cells for TNF α , GM-CSF, MIP-1 β , IL-6 and IL-8 when dosed with MPA ranging from 0nM to 390 μ M, but cultured for five days, which does not capture MPA concentrations in the drug tail [21]. Consistent with our results, IP-10 production has been demonstrated to increase with a 1-log decrease in MPA concentration, but nonetheless at high physiologic concentrations of 100nM and 10nM [5, 43]. Additionally, expression levels at supraphysiologic concentrations of MPA (10nM – 1,000nM) for IFN- γ , GM-CSF, MIP-1 β , and TNF- α in PBMCs followed a similar concentration dependent pattern [43]. IL-10 expression in ectocervical cells at closer to physiologic hormone concentrations of 0.01nM to 1nM leads to a smaller decrease in IL-10 mRNA than that between 1nM to 100nM, likely indicating that as MPA concentration approaches the physiologic range the immunosuppressive effects are reduced [18]. Furthermore, the cytokine profile of basolateral supernatants from genital epithelial cells (GECs) after MPA exposure and after viral exposure was elevated for pro-

inflammatory cytokines TNF- α , IL-1 β , GM-CSF and chemokines IL-8, IP-10, MIP-1 β [20], indicating the primary epithelial cells were producing a pro-inflammatory milieu that could promote HIV-1 infection.

With the expression of pro-inflammatory cytokines showing enhancement at physiologic concentrations of MPA [38-39], GR engagement or sexually transmitted infection (STI) exposure may be potential mechanisms influencing HIV-1 acquisition. It's been demonstrated that MPA at 1 μ M regulates IL-12 production via recruitment of the GR to the IL-12 promoter [18]. Our data show upregulation of pro-inflammatory cytokines at concentrations of 1nM and below, investigating GR engagement at these levels would provide insight whether this increase in cytokines is also mediated via the GR. Furthermore, HSV-2 seropositive populations have significantly higher HIV-1 seroconversion incidence rates (HR 4.6 [2.8–7.6]) than those who are HSV-2 negative [44-45]. This is further enhanced by DMPA use where seropositive HSV-2 DMPA users are more likely to seroconvert to HIV-1 (HR 2.01 [1.12–3.63]) than NET-EN users [10]. With approximately 50% of persons infected with HSV-2 in South African countries that also have a high HIV-1 prevalence [46], this may point to another factor driving the epidemiologic association between HIV-1 acquisition risk and DMPA use. HSV-2 increases the percentage of α 4 β 7⁺ CD4 T cells via enhancement of retinoic acid secretion by dendritic cells; this increase is typically co-upregulated with CCR5 expression and creates a highly susceptible environment for HIV-1 infection [47]. Another reason that may explain why physiologic concentrations of MPA had minimal effects on HIV-1 infection in our model could be attributed to the superficial epithelium sloughing off after 3 days in cultures [48]. A recent paper demonstrated that MPA increased epithelial permeability by reducing the expression of cell adhesion molecules and allowing entry of luminal microbiota and possibly viruses as well [33].

Some of the limitations to this work included the use of surgical cervical resections from donors with a median age of 42 years (range from 27-50). While the epidemiologic association was noted in relatively younger groups of women, progesterone receptor expression in the uterus varies during different stages of the menstrual cycle but resembles early proliferative phase levels or lower post menopause [49], which could have implications in the baseline expression level in the cervical tissues used here and its effect on receptor engagement. Furthermore, limited demographic data was collected from donors, especially those related to previous exposure to hormone therapy, chemotherapy, or biological factors preceding the surgery. Future investigations would evaluate the influence of GR engagement by incorporating a GR antagonist, for example, and integrating HSV-2 into our ex vivo model.

APPENDIX: EVALUATION OF LOW-DOSE MPA ON LOW TITER HIV-1 REPLICATION

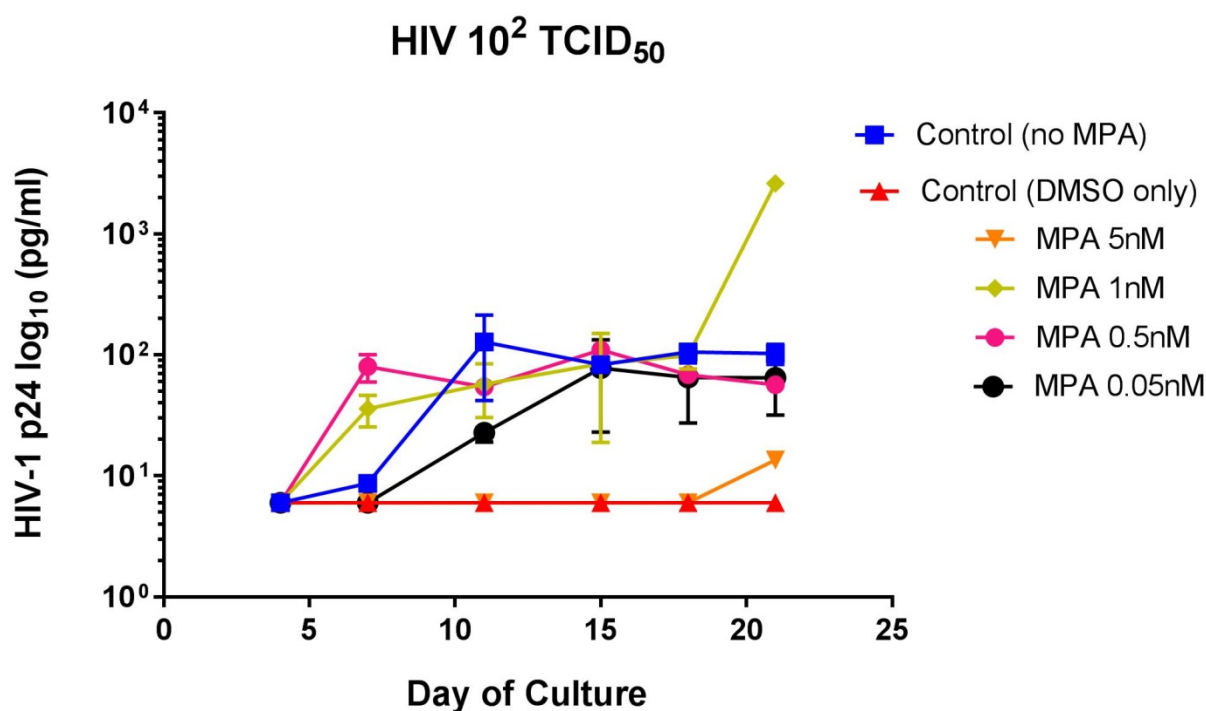


Figure 5. Appendix, Viral growth at low-dose MPA.

Viral growth and 95% CI is represented in terms of the amount of p24 for 6 collection days after incubation for 48 hours with either 5nM, 1nM, 0.5nM, or 0.05nM MPA, then infected with 5×10^2 TCID₅₀ HIV-1_{BaL} (n=1). The DMSO control in this experiment was added in concentrations comparable to the amount of DMSO added to 500μM MPA group (see Figure 2 and 3).

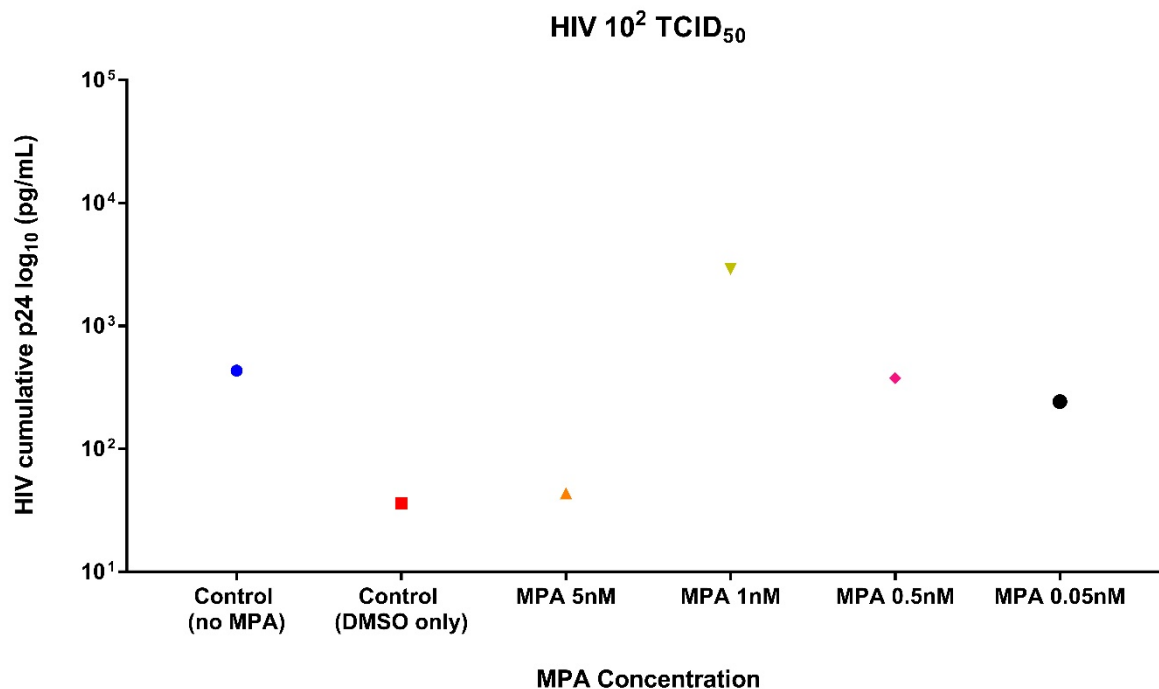


Figure 6. Appendix. Cumulative p24 at low-dose MPA.

Cumulative median p24 is represented for 6 collection days after incubation for 48 hours with either 5nM, 1nM, 0.5nM, or 0.05nM MPA, then infected with 5×10^2 TCID₅₀ HIV-1_{BaL} (n=1). The DMSO control in this experiment was added in concentrations comparable to the amount of DMSO added to 500μM MPA group (see Figure 2 and 3).

BIBLIOGRAPHY

1. Kaunitz, AM. Depot medroxyprogesterone acetate for contraception. In: UpToDate, Topic 5468 (Version 48.0), UpToDate, Waltham, MA. (Accessed on January 10, 2017.)
2. Chelsea B. Polis, S. J. (2014). Hormonal contraceptive methods and risk of HIV acquisition in women. *Contraception*, 90(4), 360-390. <http://dx.doi.org/10.1016/j.contraception.2014.07.009>
3. Chelsea B. Polis, K. M. (2016). An updated systematic review of epidemiological. *AIDS*, 30(17), 2665-2683. doi:10.1097/QAD.0000000000001228
4. Heffron, R., Donnell, D., Rees, H., Celum, C., Mugo, N., Were, E., Baeten, J. M. (2012). Hormonal contraceptive use and risk of HIV-1 transmission: a prospective cohort analysis. *The Lancet Infectious Diseases*, 12(1), 19–26. [http://doi.org/10.1016/S1473-3099\(11\)70247-X](http://doi.org/10.1016/S1473-3099(11)70247-X)
5. Huijbregts RP, Michel KG, Hel Z. Effect of progestins on immunity: medroxyprogesterone but not norethisterone or levonorgestrel suppresses the function of T cells and pDCs. *Contraception*. 2014 Aug;90(2):123-9. doi: 10.1016/j.contraception.2014.02.006. PubMed PMID: 24674041; PubMed Central PMCID: PMC4874781
6. Guthrie, B. L., Introini, A., Roxby, A. C., Choi, R. Y., Bosire, R., Lohman-Payne, B., Broliden, K. (2015). Depot Medroxyprogesterone Acetate (DMPA) Use is Associated with Elevated Innate Immune Effector Molecules in Cervicovaginal Secretions of HIV-1-uninfected Women. *Journal of Acquired Immune Deficiency Syndromes* (1999), 69(1), 1–10. <http://doi.org/10.1097/QAI.0000000000000533>.
7. Hormonal contraceptive methods for women at high risk of HIV and living with HIV: 2014 guidance statement. (2014). Retrieved January 15, 2017, from http://apps.who.int/iris/bitstream/10665/128537/1/WHO_RHR_14.24_eng.pdf?ua=1.
8. United Nations, Department of Economic and Social Affairs, Population Division (2015). Trends in Contraceptive Use Worldwide 2015. Annex Table II. (ST/ESA/SER.A/349).
9. Getting to Zero: HIV in Eastern and Southern Africa. UNAIDS, 2013.

10. Noguchi LM, Richardson BA, Baeten JM, Hillier SL, Balkus JE, Chirenje ZM, Bunge K, Ramjee G, Nair G, Palanee-Phillips T, Selepe P, van der Straten A, Parikh UM, Gomez K, Piper JM, Watts DH, Marrazzo JM; VOICE Study Team. Risk of HIV-1 acquisition among women who use different types of injectable progestin contraception in South Africa: a prospective cohort study. *Lancet HIV*. 2015 Jul;2(7):e279-87. PubMed PMID: 26155597; PubMed Central PMCID: PMC4491329.
11. Kaushic C, Ferreira VH, Kafka JK, Nazli A. HIV infection in the female genital tract: discrete influence of the local mucosal microenvironment. *Am J Reprod Immunol*. 2010 Jun;63(6):566-75. doi: 10.1111/j.1600-0897.2010.00843.x. Review. PubMed PMID: 20384619.
12. Pudney J, Quayle AJ, Anderson DJ. Immunological microenvironments in the human vagina and cervix: mediators of cellular immunity are concentrated in the cervical transformation zone. *Biol Reprod*. 2005 Dec;73(6):1253-63. PubMed PMID: 16093359
13. Halpern V, Combes SL, Dorflinger LJ, Weiner DH, Archer DF. Pharmacokinetics of subcutaneous depot medroxyprogesterone acetate injected in the upper arm. *Contraception*. 2014 Jan;89(1):31-5. doi: 10.1016/j.contraception.2013.07.002. PubMed PMID: 23993431.
14. Sciaranghella, G., Wang, C., Hu, H., Anastos, K., Merhi, Z., Nowicki, M., Tsibris, A. M. N. (2015). CCR5 Expression Levels in HIV-Uninfected Women Receiving Hormonal Contraception. *The Journal of Infectious Diseases*, 212(9), 1397–1401. <http://doi.org/10.1093/infdis/jiv233>
15. Patterson, B. K., Landay, A., Andersson, J., Brown, C., Behbahani, H., Jiyamapa, D., Garcia, P. (1998). Repertoire of Chemokine Receptor Expression in the Female Genital Tract : Implications for Human Immunodeficiency Virus Transmission. *The American Journal of Pathology*, 153(2), 481–490.
16. Joag VR, McKinnon LR, Liu J, Kidane ST, Yudin MH, Nyanga B, Kimwaki S, Besel KE, Obila JO, Huibner S, Oyugi JO, Arthos J, Anzala O, Kimani J, Ostrowski MA; Toronto HIV Research Group., Kaul R. Identification of preferential CD4+ T-cell targets for HIV infection in the cervix. *Mucosal Immunol*. 2016 Jan;9(1):1-12. doi: 10.1038/mi.2015.28. PubMed PMID: 25872482.
17. Koubovec D, Ronacher K, Stubbsrud E, Louw A, Hapgood JP. Synthetic progestins used in HRT have different glucocorticoid agonist properties. *Mol Cell Endocrinol*. 2005 Oct 20;242(1-2):23-32. PubMed PMID: 16125839.
18. Louw-du Toit R, Hapgood JP, Africander D. Medroxyprogesterone acetate differentially regulates interleukin (IL)-12 and IL-10 in a human ectocervical epithelial cell line in a glucocorticoid receptor (GR)-dependent manner. *J Biol Chem*. 2014 Nov 7;289(45):31136-49. doi: 10.1074/jbc.M114.587311. PubMed PMID: 25202013; PubMed Central PMCID: PMC4223317.

19. Africander DJ, Storbeck KH, Hapgood JP. A comparative study of the androgenic properties of progesterone and the progestins, medroxyprogesterone acetate (MPA) and norethisterone acetate (NET-A). *J Steroid Biochem Mol Biol*. 2014 Sep;143:404-15. doi: 10.1016/j.jsbmb.2014.05.007. PubMed PMID: 24861265.
20. Ferreira VH, Dizzell S, Nazli A, Kafka JK, Mueller K, Nguyen PV, Tremblay MJ, Cochrane A, Kaushic C. Medroxyprogesterone Acetate Regulates HIV-1 Uptake and Transcytosis but Not Replication in Primary Genital Epithelial Cells, Resulting in Enhanced T-Cell Infection. *J Infect Dis*. 2015 Jun 1;211(11):1745-56. doi: 10.1093/infdis/jiu832.
21. Irvin SC, Herold BC. Molecular mechanisms linking high dose medroxyprogesterone with HIV-1 risk. *PLoS One*. 2015 Mar 23;10(3):e0121135. doi: 10.1371/journal.pone.0121135.
22. Hormonal Contraception and HIV: Technical Statement. Geneva: World Health Organization; 2012 Feb 16. PubMed PMID: 23586117.
23. Hormonal Contraception and HIV: Guidance Statement. Recommendations concerning the use of hormonal contraceptive methods by women at high risk of HIV. World Health Organization; 2017. WHO reference number: WHO/RHR/17.04.
24. Jain, A. K. (2012). Hormonal contraception and HIV acquisition risk: implications for individual users and public policies. *Contraception*, 86(6), 645-652. doi:10.1016/j.contraception.2012.03.008.
25. McCoy SI, Zheng W, Montgomery ET, Blanchard K, van der Straten A, de Bruyn G, Padian NS. Oral and injectable contraception use and risk of HIV acquisition among women in sub-Saharan Africa. *AIDS*. 2013 Mar 27;27(6):1001-9. doi: 10.1097/QAD.0b013e32835da401. PubMed PMID: 23698064.
26. Kleinschmidt I, Rees H, Delany S, Smith D, Dinat N, Nkala B, McIntyre JA. Injectable progestin contraceptive use and risk of HIV infection in a South African family planning cohort. *Contraception*. 2007 Jun;75(6):461-7. PubMed PMID: 17519153.
27. Schindler AE, Campagnoli C, Druckmann R, Huber J, Pasqualini JR, Schweppe KW, Thijssen JH. Classification and pharmacology of progestins. *Maturitas*. 2003 Dec 10;46 Suppl 1:S7-S16. Review. PubMed PMID: 14670641.
28. Govender, Y., Avenant, C., Verhoog, N. J., Ray, R. M., Grantham, N. J., Africander, D., & Hapgood J. P. (2014). The injectable-only contraceptive medroxyprogesterone acetate, unlike norethisterone acetate and progesterone, regulates inflammatory genes in endocervical cells via the glucocorticoid receptor. *PLoS One*, 9(5), e96497. doi:10.1371/journal.pone.0096497
29. Hapgood, J. P., Ray, R. M., Govender, Y., Avenant, C., & Tomasicchio, M. (2014). Differential glucocorticoid receptor-mediated effects on immunomodulatory gene

expression by progestin contraceptives: implications for HIV-1 pathogenesis. *Am J Reprod Immunol*, 71(6), 505-512. doi:10.1111/aji.12214

30. Francis, S. C., Hou, Y., Baisley, K., van de Wijgert, J., Watson-Jones, D., Ao, T. T., Shattock, R. J. (2016). Immune Activation in the Female Genital Tract: Expression Profiles of Soluble Proteins in Women at High Risk for HIV Infection. *PLoS ONE*, 11(1), e0143109. <http://doi.org/10.1371/journal.pone.0143109>
31. Wilson, S. S., Wiens, M. E., & Smith, J. G. (2013). Antiviral Mechanisms of Human Defensins. *Journal of Molecular Biology*, 425(24), 10.1016/j.jmb.2013.09.038. <http://doi.org/10.1016/j.jmb.2013.09.038>
32. Valere, K., Rapista, A., Eugenin, E., Lu, W., & Chang, T. L. (2015). Human Alpha-Defensin HNP1 Increases HIV Traversal of the Epithelial Barrier: A Potential Role in STI-Mediated Enhancement of HIV Transmission. *Viral Immunology*, 28(10), 609–615. <http://doi.org/10.1089/vim.2014.0137>
33. Bergman, P., Walter-Jallow, L., Broliden, K., Agerberth, B., & Soderlund, J. (2007). The antimicrobial peptide LL-37 inhibits HIV-1 replication. *Curr HIV Res*, 5(4), 410-415.
34. Ogawa, Y., Kawamura, T., Matsuzawa, T., Aoki, R., Gee, P., Yamashita, A., Shimada, S. (2013). Antimicrobial peptide LL-37 produced by HSV-2-infected keratinocytes enhances HIV infection of Langerhans cells. *Cell Host Microbe*, 13(1), 77-86. doi:10.1016/j.chom.2012.12.002
35. Chandra, N., Thurman, A. R., Anderson, S., Cunningham, T. D., Yousefieh, N., Mauck, C., & Doncel, G. F. (2013). Depot medroxyprogesterone acetate increases immune cell numbers and activation markers in human vaginal mucosal tissues. *AIDS Res Hum Retroviruses*, 29(3), 592-601. doi:10.1089/aid.2012.0271
36. Calla, N. E. Q., Miguel, R. D. V., Boyaka, P. N., Hall-Stoodley, L., Kaur, B., Trout, W., Cherpes, T. L. (2016). Medroxyprogesterone acetate and levonorgestrel increase genital mucosal permeability and enhance susceptibility to genital herpes simplex virus type 2 infection. *Mucosal Immunology*, 9(6), 1571–1583. <http://doi.org/10.1038/mi.2016.22>
37. Shannon, B., Yi, T. J., Thomas-Pavanel, J., Chieza, L., Janakiram, P., Saunders, M., Kaul, R. (2014). Impact of asymptomatic herpes simplex virus type 2 infection on mucosal homing and immune cell subsets in the blood and female genital tract. *J Immunol*, 192(11), 5074-5082. doi:10.4049/jimmunol.1302916
38. Rollenhagen, C., Lathrop, M. J., Macura, S. L., Doncel, G. F., & Asin, S. N. (2014). Herpes simplex virus type-2 stimulates HIV-1 replication in cervical tissues: implications for HIV-1 transmission and efficacy of anti-HIV-1 microbicides. *Mucosal Immunology*, 7(5), 1165–1174. <http://doi.org/10.1038/mi.2014.3>
39. Desai, D. V., & Kulkarni, S. S. (2015). Herpes Simplex Virus: The Interplay Between HSV, Host, and HIV-1. *Viral Immunol*, 28(10), 546-555. doi:10.1089/vim.2015.0012

40. Polis, C. B., Westreich, D., Balkus, J. E., Heffron, R., & participants of the 2013 HC-HIV observational analysis meeting. (2013). Assessing the effect of hormonal contraception on HIV acquisition in observational data: challenges and recommended analytic approaches. *AIDS* (London, England), 27(0 1), S35–S43. <http://doi.org/10.1097/QAD.0000000000000036>
41. FHI 360. (2016, December). The Evidence for Contraceptive Options and HIV Outcomes Trial (ECHO). Retrieved March 20, 2017, from <https://clinicaltrials.gov/ct2/show/NCT02550067>.
42. Sampah, M. E. S., Laird, G. M., Blankson, J. N., Siliciano, R. F., & Coleman, J. S. (2015). Medroxyprogesterone acetate increases HIV-1 infection of unstimulated peripheral blood mononuclear cells in vitro. *AIDS* (London, England), 29(10), 1137–1146. <http://doi.org/10.1097/QAD.0000000000000681>
43. Huijbregts, R. P. H., Helton, E. S., Michel, K. G., Sabbaj, S., Richter, H. E., Goepfert, P. A., & Hel, Z. (2013). Hormonal Contraception and HIV-1 Infection: Medroxyprogesterone Acetate Suppresses Innate and Adaptive Immune Mechanisms. *Endocrinology*, 154(3), 1282–1295. <http://doi.org/10.1210/en.2012-1850>
44. Baeten, J. M., Benki, S., Chohan, V., Lavreys, L., McClelland, R. S., Mandaliya, K., Overbaugh, J. (2007). Hormonal contraceptive use, herpes simplex virus infection, and risk of HIV-1 acquisition among Kenyan women. *Aids*, 21(13), 1771-1777. doi:10.1097/QAD.0b013e328270388a
45. Brown, J. M., Wald, A., Hubbard, A., Rungruenthanakit, K., Chipato, T., Rugpao, S., Padian, N. S. (2007). Incident and prevalent herpes simplex virus type 2 infection increases risk of HIV acquisition among women in Uganda and Zimbabwe. *Aids*, 21(12), 1515-1523. doi:10.1097/QAD.0b013e3282004929
46. WHO/UN AIDS. Herpes Simplex Virus Type 2: Programmatic and Research Priorities in Developing Countries. Geneva: World health Organization; 2001. http://www.who.int/hiv/pub/sti/en/hiv_aids_2001.05.pdf.
47. Martinelli, E., Tharinger, H., Frank, I., Arthos, J., Piatak, M., Jr., Lifson, J. D., Robbani, M. (2011). HSV-2 Infection of Dendritic Cells Amplifies a Highly Susceptible HIV-1 Cell Target. *PLOS Pathogens*, 7(6), e1002109. doi:10.1371/journal.ppat.1002109
48. Cummins, J. E., Guarner, J., Flowers, L., Guenther, P. C., Bartlett, J., Morken, T., Dezzutti, C. S. (2007). Preclinical Testing of Candidate Topical Microbicides for Anti-Human Immunodeficiency Virus Type 1 Activity and Tissue Toxicity in a Human Cervical Explant Culture . *Antimicrobial Agents and Chemotherapy*, 51(5), 1770–1779. <http://doi.org/10.1128/AAC.01129-06>
49. Snijders, M. P., de Goeij, A. F., Debets-Te Baerts, M. J., Rousch, M. J., Koudstaal, J., & Bosman, F. T. (1992). Immunocytochemical analysis of oestrogen receptors and progesterone receptors in the human uterus throughout the menstrual cycle and after the menopause. *J Reprod Fertil*, 94(2), 363-371.